PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL

MICHELE LUNARDI

CARACTERIZAÇÃO MOLECULAR DO GENE L1 DE UM PROVÁVEL NOVO TIPO DE PAPILOMAVÍRUS BOVINO IDENTIFICADO NO BRASIL

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Dissertação apresentada para a obtenção do título de Mestre em Ciência Animal (Área de Concentração: Sanidade Animal) da Universidade Estadual de Londrina.

Orientador: Prof. Dr. Amauri Alcindo Alfieri

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RESUMO

LUNARDI, M. **Caracterização molecular do gene L1 de um provável novo tipo de papilomavírus bovino identificado no Brasil.** 2008. 81f. Dissertação (Mestrado em Ciência Animal, Área de Concentração: Sanidade Animal) – Universidade Estadual de Londrina, Londrina. 2008.

O papilomavírus (PV) constitui um grupo diverso de pequenos oncovírus não-envelopados e com genoma DNA fita dupla circular, classificados na família *Papillomaviridae*. Em bovinos são descritos 10 tipos de papilomavírus bovino (BPV) que, com base na identidade de nucleotídeos da proteína estrutural L1, estão distribuídos nos gêneros *Deltapapillomavirus* (BPV-1 e -2), *Epsilonpapillomavirus* (BPV-5 e -8), *Xipapillomavirus* (BPV-3, -4, -6, -9 e -10) e em um gênero ainda não definido (BPV-7). A infecção por diversos tipos de BPV tem sido relacionada a diferentes quadros clínicos em bovinos, destacando-se a papilomatose cutânea. A identificação dos tipos virais que determinam sinais clínicos e daqueles que resultam em infecções subclínicas tem sido realizada por meio da técnica da PCR, tanto em bovinos quanto em seres humanos. Como a L1 é a proteína mais conservada entre os PVs, foram desenvolvidos oligonucleotídeos iniciadores (*primers*) consensuais e genéricos, que apresentam alta identidade de nucleotídeos com seqüências conservadas da ORF L1. Esses sistemas de PCR têm sido utilizados para detectar uma grande variedade de tipos virais em amostras clínicas. O objetivo deste trabalho foi estabelecer o posicionamento filogenético de um provável novo tipo de BPV (BPV/BR-UEL2), identificado no Brasil, por meio da caracterização molecular de seu gene L1. O DNA do BPV/BR-UEL2 foi isolado a partir de um papiloma cutâneo, localizado na região axilar de uma vaca leiteira do Estado do Paraná. Como a análise anterior, envolvendo a seqüência de 475 pb obtida pela PCR utilizando os *primers* FAP59/FAP64, havia indicado que o BPV/BR-UEL2 era mais relacionado ao BPV tipo 4, os alinhamentos das regiões genômicas L2, L1 e LCR de alguns representantes do gênero *Xipapillomavirus* (BPV-3, -4 e -6) foram utilizados na elaboração de *primers* genéricos. Adicionalmente, o par de *primers* FAP também foi empregado, tanto na sua forma original quanto em combinações com os *primers* desenhados para este estudo. O primeiro segmento do gene L1 pôde ser obtido por meio de um sistema *semi-nested* (SN-PCR) empregando na primeira etapa de amplificação os *primers* L2Bf/FAP64, e o par de *primers* L2Bf/L1Br na segunda etapa de amplificação, resultando em um produto de PCR de 435 pb. A amplificação das regiões remanescentes do mesmo gene foi obtida a partir dos *primers* FAP59/FAP64 (475 pb) e L1Bf/LCRBr (1128 pb). Os referidos produtos de PCR foram posteriormente submetidos à clonagem e seqüenciamento. A análise filogenética envolvendo seqüências completas da ORF L1 revelou que a amostra BPV/BR-UEL2 estava relacionada aos tipos de BPV agrupados no gênero *Xipapillomavirus*. O provável novo tipo de BPV analisado neste estudo apresentou maior similaridade (78%) com a seqüência de nucleotídeos do gene L1 do BPV tipo 4, o que sugere a sua classificação no gênero *Xipapillomavirus*. No Brasil, apesar do caráter endêmico das infecções pelo BPV, a identificação dos tipos de BPV em rebanhos bovinos ainda é esporádica. Recentemente, a utilização do par de *primers* FAP59/FAP64 permitiu a identificação de quatro prováveis novos tipos virais, ainda não descritos no mundo, e provenientes de bovinos do estado do Paraná. No presente estudo, o posicionamento filogenético de um destes tipos virais, detectado a partir de uma lesão cutânea de uma vaca leiteira, foi determinado. A realização de estudos complementares envolvendo a epidemiologia molecular das infecções pelo BPV, tanto em rebanhos bovinos brasileiros quanto de diversas regiões geográficas ao redor do mundo, poderiam indicar a prevalência e checar a associação deste isolado com lesões cutâneas.

Palavras-chave: Bovino, Papilomatose cutânea, BPV, Provável novo tipo de BPV, Gene L1, Análise filogenética.

ABSTRACT

LUNARDI, M. **Molecular characterization of L1 gene of a putative new BPV type identified in Brazil.** 2008. 81f. Dissertation (Master's Degree in Animal Science) - Universidade Estadual de Londrina, Londrina. 2008.

Papillomaviruses (PVs) comprise a highly diverse group of non-enveloped oncovirus classified in *Papillomaviridae* family, whose genome consists of a circular double-stranded DNA molecule. Depending on nucleotide identity presented in structural protein L1, 10 types of bovine papillomavirus (BPV) are described in cattle, being each of them located in genera *Deltapapillomavirus* (BPV-1 and -2), *Epsilonpapillomavirus* (BPV-5 and -8), *Xipapillomavirus* (BPV-3, -4, -6, -9, and -10), and an yet assigned genus (BPV-7). Infection by diverse BPV types has been associated with different clinical outcomes in cattle, being the cutaneous papillomatosis considered as an important cause of economic losses. The identification of viral types involved with either clinical manifestations or assintomatic infections, has been mainly performed by PCR assay, both in cattle and humans. Since the L1 is considered as the most conserved protein in PVs, consensus and degenerate *primers*, manifesting a high degree of nucleotide identity with sequences in L1 ORF, has been designed to detect a broad range of viral types in clinical specimens. The aim of the current study is to state the phylogenetic position of a previously identified putative new BPV type (BPV/BR-UEL2) detected in Paraná state, Brazil, through the molecular characterization of its L1 gene. The BPV/BR-UEL2 was isolated from a papilloma located in the axillary region of a dairy cow. As the previous FAP sequence analysis had revealed our isolate as closest related to BPV type 4, alignments of L1, L2, LCR genomic regions of some *Xipapillomavirus* representatives (BPV-3, -4, and -6), were used on design of degenerate primers. In addition, the previously described FAP primer pair was also employed both in the original form as in combination with the designed primers. The first L1 segment of the Brazilian isolate could be achieved by a semi-nested PCR system (SN-PCR) employing L2Bf/FAP64 primers in the first round, and L2Bf/L1Br primer pair in the second round, which yielded an amplicon of 435 bp. The use of the FAP59/FAP64 (475 bp) and L1Bf/LCRBr (1128 bp) primer sets allowed the amplification of the remaining portions of the same gene. These three overlapping amplicons obtained from the L1 ORF were submitted to cloning and then sequenced. Phylogenetic analysis with complete L1 ORF sequences revealed the BPV/BR-UEL2 isolate as related with BPV types held in *Xipapillomavirus* genus, displaying the highest L1 nucleotide sequence similarity with BPV type 4 (78%), what suggests its classification in the *Xipapillomavirus* genus. In Brazil, despite the relatively common occurrence of BPV infections, the identification of BPV types in cattle herds is still sporadic. Recently, the use of the FAP59/FAP64 primer pair enabled the identification of four putative new BPV types, not yet described around the world, from cattle from Parana state. In the present study, we determine the phylogenetic position of one of these viral types, which was detected from a cutaneous lesion of a dairy cow. The realization of further studies involving the molecular epidemiology of BPV infections, in Brazilian cattle herds as much in diverse geographical areas around the world, could indicate its prevalence throughout the cattle as well as check its association with cutaneous lesions.

Key Words: Bovine, Cutaneous papillomatosis, BPV, Putative new BPV type, L1 gene, Phylogenetic analysis.

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1. REVISÃO DE LITERATURA

1. REVISÃO DE LITERATURA

O papilomavírus (PV – *papillomavirus*) constitui um grupo de vírus DNA altamente diverso, tendo sido identificado em distintas espécies de mamíferos e de aves (De Villiers et al., 2004). Além dos seres humanos, os PVs foram encontrados em diversas espécies: caprinos (ChPV-1); ovinos (OvPV-1); cervídeos (DPV – cervos; EEPV – alces); eqüídeos (EcPV-1); primatas não-humanos (RhPV-1 – macaco rhesus); felinos (FdPV – gato doméstico); sirénios (TmPV-1 – peixe-boi-marinho); cetáceos (PsPV – boto-de-burmeister) e aves (PePV – papagaio africano cinzento) (Stenlund et al., 1983; Groff e Lancaster, 1985; Ostrow et al., 1991; Tachezy et al., 2002; Terai e Burk, 2002; Ghim et al., 2004; Narechania et al., 2004; Rector et al., 2004; Van Doorslaer et al., 2006; Van Bressem et al., 2007).

Nos seres humanos, a única espécie hospedeira cuja infecção pelo papilomavírus tem sido bem estudada, cerca de 100 tipos diferentes de papilomavírus (HPV – *human papillomavirus*) foram descritos por meio da determinação da seqüência completa do genoma viral. Porém, dados obtidos a partir de seqüências de produtos subgenômicos têm evidenciado a existência de outros 100 supostos novos tipos virais (Bernard, 2005).

O PV é um vírus epiteliotrópico com potencial para a indução de lesões benignas tanto na pele (papilomas ou verrugas) quanto em mucosas (condilomas) de seus hospedeiros naturais. Entretanto, também tem sido atribuído a alguns PVs o desenvolvimento de lesões epiteliais malignas, especialmente em casos de câncer do colo uterino e outros tumores do trato urogenital. O reconhecimento de que alguns tipos específicos de HPVs representam importantes patógenos para os seres humanos, uma vez que estão diretamente associados a diferentes tumores, tem justificado sua importância médica e estimulado esforços no desenvolvimento de vacinas contra este vírus (Howley e Lowy, 2001).

A infecção por diversos tipos de papilomavírus bovino (BPV – *bovine papillomavirus*) tem sido relacionada a diferentes quadros clínicos em bovinos. A ocorrência de tumores cutâneos benignos, caracterizando a papilomatose cutânea, pode ser verificada em diversas regiões do corpo do animal. Na dependência da extensão das lesões, há o comprometimento do desenvolvimento corporal dos animais, predisposição a infecções e/ou infestações secundárias, depreciação do couro, entre outras conseqüências que podem acarretar prejuízos econômicos à exploração pecuária de corte e, principalmente, leiteira. Papilomas localizados no úbere e nos tetos de vacas em lactação dificultam a amamentação dos bezerros, a ordenha tanto manual quanto, principalmente, mecânica e, devido às infecções bacterianas secundárias, predispõem a mastites clínicas e/ou subclínicas ascendentes (Campo, 2002).

A interação entre tipos específicos do BPV e a ingestão prolongada da planta samambaia (*Pteridium aquilinum*), tem sido apontada como a etiologia tanto da hematúria enzoótica quanto do câncer do trato gastrointestinal superior em bovinos. No primeiro caso, acredita-se que inicialmente ocorra uma infecção latente pelo BPV-2 na mucosa da bexiga. Posteriormente, a infecção seria reativada e induzida à neoplasia por meio de compostos químicos imunossupressivos e carcinogênicos presentes na samambaia, culminando na progressão à malignidade. Quanto aos tumores do canal alimentar, a imunossupressão causada pela ingestão da samambaia define a persistência dos papilomas, induzidos pelo BPV-4, que por ação de elementos carcinogênicos da planta podem evoluir para carcinomas malignos (Campo et al., 1994, 1997; Borzacchiello et al., 2003).

A associação do PV com diversos tipos de tumores em várias espécies de hospedeiros já está bem caracterizada. Contudo, estudos com o objetivo de avaliar a presença do HPV em pele saudável, tanto em grupos de pacientes imunossuprimidos quanto de voluntários imunocompetentes e saudáveis, têm revelado a ocorrência de infecções subclínicas na maioria dos indivíduos de ambos os grupos. A elevada freqüência do vírus e a grande multiplicidade

de tipos virais demonstrada em pele saudável têm sugerido que o HPV possa estar presente, como um agente comensal, na pele da população em geral (Boxman et al., 1997; Astori et al., 1998; Antonsson et al., 2000).

O PV é espécie-específico e, até mesmo em condições experimentais, não é capaz de infectar outra espécie que não o seu hospedeiro natural. O único caso conhecido de infecção inter-espécies é o sarcóide eqüino, que é a infecção de eqüídeos pelos BPV-1 e BPV-2 (Campo, 2002).

Devido à não existência de outros casos relatados de quebra da barreira inter-espécies com relação à infecção pelo PV, até o momento, não foi obtido um modelo animal susceptível à infecção pelo HPV. Com isso, alguns modelos experimentais de infecção com PV animal têm sido cruciais na investigação da biologia do vírus, da sua relação com o hospedeiro, da resposta imune contra o vírus e no desenvolvimento das primeiras vacinas contra PV. Esses modelos animais têm balizado os estudos conduzidos com o HPV, tanto no seu envolvimento direto com neoplasias, quanto na relação entre o vírus e co-carcinógenos ambientais, primeiramente estabelecidos com PV animal, particularmente o papilomavírus dos coelhos-americanos (CRPV – *cottontail rabbit papillomavirus*), o BPV, e o papilomavírus oral canino (COPV – *canine oral papillomavirus*) (Campo, 2002).

O PV tem 52 a 55 nm de diâmetro, é desprovido de envelope lipoprotéico, e apresenta simetria icosaédrica. A replicação viral ocorre no núcleo de células epiteliais pavimentosas. O vírion é constituído de uma única molécula de DNA, fita dupla e circular, com aproximadamente 8000 pares de base (pb), contida em um capsídeo composto de 72 capsômeros (Crawford e Crawford, 1963).

A organização genômica dos diversos PVs é muito semelhante. Uma característica compartilhada pelos PVs é o fato de todos os quadros abertos de leitura (ORFs – *open reading frames*) estarem contidos em uma das fitas do DNA viral, indicando que todos os genes são

codificados em apenas uma fita. Estudos sobre a transcrição dos RNAs codificados pelos PVs, têm confirmado que somente uma das fitas desempenha a função de molde na transcrição. A fita codificante é constituída por até 10 ORFs que são classificadas, com base em sua localização no genoma viral, em iniciais (E – *early*) e tardias (L – *late*). O segmento inicial do genoma (E) pode ser constituído por até oito ORFs, enquanto o segmento final (L) geralmente contém duas ORFs. Entre estes segmentos existe ainda uma região desprovida de ORFs e denominada LCR (*long control region*), onde estão presentes tanto a origem de replicação quanto diversos promotores (Howley e Lowy, 2001).

A expressão das seis proteínas não-estruturais e regulatórias mais comuns (E1, E2, E4, E5, E6 e E7), codificadas pela região inicial do genoma viral, ocorre em queratinócitos indiferenciados ou em etapas intermediárias de diferenciação. A expressão das duas proteínas estruturais virais (L1 e L2), codificadas pelo segmento final do genoma, ocorre em queratinócitos em fase final de diferenciação (Zheng e Baker, 2006).

As proteínas não-estruturais E1 e E2 estão envolvidas na replicação do DNA viral e na regulação do início da transcrição. A E4, que é expressa em infecções produtivas, está associada com o colapso dos filamentos de citoqueratina, enquanto E5, E6 e E7 constituem oncogenes virais, cuja expressão induz a imortalização e a transformação das células infectadas (Zheng e Baker, 2006).

O capsídio viral é constituído pelas duas proteínas estruturais (L1 e L2). A principal proteína do capsídio (L1) tem aproximadamente 55 kD e representa cerca de 80% da quantidade total de proteína do vírus. Por outro lado, a proteína L2 tem tamanho molecular aproximado de 70 kD (Frave et al., 1975). Além disso, sabe-se que partículas semelhantes ao vírus (VLP – *virus-like particles*) podem ser produzidas a partir de diferentes PVs somente pela expressão da L1 ou pela combinação da L1 com a L2, utilizando tanto sistemas de expressão protéica de mamíferos quanto outros (Rose et al., 1993; Zhou et al., 1993). Embora

a L2 não seja necessária para o processo de montagem viral, ela é incorporada em VLPs quando co-expressa com a L1. Quando visualizadas por microscopia crioeletrônica, a morfologia das VLPs, contendo somente a L1, parece ser idêntica à das partículas virais intactas (Hagensee et al., 1994). Os epítopos que induzem a produção de anticorpos neutralizantes estão presentes principalmente na L1, sendo também localizados na L2 (Roden et al., 1994).

Originalmente, o PV foi agrupado juntamente com o poliomavírus para formar a família *Papovaviridae*. Porém, como estes dois grupos de vírus apresentam diferenças no tamanho e na organização genômica, e nenhuma similaridade significativa quanto às seqüências de nucleotídeos e aminoácidos, eles são atualmente reconhecidos pelo Comitê International de Taxonomia Viral como duas famílias distintas, com os PVs constituindo a família *Papillomaviridae* (De Villiers et al., 2004).

Diferentemente da taxonomia de outras famílias virais, a introdução formal de níveis taxonômicos tais como "gênero" e "espécie", ocorreu apenas recentemente na família *Papillomaviridae* (De Villiers et al., 2004). Assim, como o estabelecido de uma forma geral em biologia, um gênero específico de PV une espécies filogeneticamente relacionadas, mas que, com freqüência, diferem quanto às características biológicas (figura 1). Já uma espécie agrupa tipos de PV que apresentam tanto relação filogenética próxima quanto propriedades biológicas e patológicas comuns. A tradução destas relações taxonômicas para a identidade observada entre seqüências de nucleotídeos ocorre da seguinte forma: i) para os diferentes gêneros a similaridade na ORF L1 é inferior a 60%. Quando a comparação é realizada utilizando seqüências genômicas completas é necessária similaridade superior a 23%, porém inferior a 43%; ii) Similaridades na ORF L1 entre 60 e 70% definem diferentes espécies virais.

Em relação ao termo "tipo", que é tradicionalmente utilizado na descrição de novos isolados de PV, utiliza-se a definição estabelecida no Encontro Internacional de Papilomavírus, realizado em 1995. Como critério preconiza-se que um novo tipo de PV é reconhecido quando o seu genoma completo for clonado e a seqüência de nucleotídeos da ORF L1 diferir em mais de 10% do tipo de PV mais próximo a ele e já anteriormente descrito. A ocorrência de diferenças, na mesma ORF, entre 2 e 10% definem um subtipo, enquanto as diferenças menores de 2% caracterizam uma variante viral (De Villiers et al., 2004). Quando a descrição de um novo isolado de PV é realizada por meio de uma seqüência parcial da ORF L1, ele é definido como "provável novo tipo viral" (Antonsson et al., 2003; Ogawa et al., 2004).

Devido à inexistência de sistemas convencionais de cultivo celular para a manutenção dos PVs e também pela extensa reatividade sorológica entre eles, a identificação dos tipos virais envolvidos nos diversos quadros clínicos tem sido realizada por meio de técnicas que identificam o DNA viral (Lowy e Howley, 2001). Considerando a grande correlação entre os HPVs e distintos tumores em humanos, o interesse por uma identificação adequada dos tipos virais tem aumentado consideravelmente.

Desde a constatação da ocorrência de grande diversidade nos HPVs, a reação em cadeia pela polimerase (PCR – *polymerase chain reaction*) tem sido apontada como o método de escolha para a identificação do DNA de PV em espécimes clínicos. Como a L1 é a proteína mais conservada entre os PVs, oligonucleotídeos iniciadores (*primers*) direcionados para o gene L1 tem sido utilizados, com freqüência, para a detecção de HPVs (Gravitt e Manos, 1992).

Em humanos, técnicas de PCR utilizando *primers* consensuais e genéricos, que apresentam alta identidade de nucleotídeos com seqüências conservadas das ORFs E1 e, principalmente, L1, têm sido desenvolvidas. Esses sistemas de PCR têm sido rotineiramente

utilizados para detectar uma grande variedade de tipos de HPV em amostras clínicas provenientes de pele ou mucosas (Manos et al., 1989; Forslund et al., 1999). Tanto o seqüenciamento direto dos produtos da PCR quanto a clonagem e posterior seqüenciamento constituem metodologias comumente utilizadas na genotipagem dos HPVs presentes em espécimes clínicos. Esta estratégia tem se mostrado promissora na obtenção de seqüências de tipos de HPV ainda não caracterizados, assim como na identificação de mutações entre os tipos já conhecidos (Forslund et al., 1999; Chow et al., 2000; Huang et al., 2004).

Atualmente, dois diferentes sistemas de PCR, que envolvem o uso de *primers* genéricos inicialmente desenhados para a amplificação do HPV, têm sido aplicados em estudos que abordam a diversidade do BPV. O primeiro deles, empregando o par de *primers* MY09/MY11, foi originalmente desenvolvido para detectar alguns tipos específicos de HPV relacionados com infecções em mucosas. Porém, esse sistema também tem possibilitado a amplificação da extremidade 3' do gene L1 da maioria dos HPVs genitais (Manos et al., 1989; Bernard et al., 1994). O outro sistema, que emprega o par de *primers* FAP59/FAP64, foi primeiramente delineado para amplificar um fragmento na extremidade 5' da ORF L1 de tipos de HPV cutâneos. Contudo, esse sistema também permitiu a detecção de um grande número de tipos de HPV, tanto cutâneos quanto genitais (Forslund et al., 1999; Antonsson et al., 2000, 2003).

Enquanto centenas de tipos de HPVs já foram definidos, até o início da década de 1980 apenas seis tipos de BPV (BPV-1 a BPV-6) tinham sido identificados a partir de casos de papilomatose cutânea e câncer em bovinos (Pfister et al., 1979; Campo et al., 1980, 1981; Campo e Coggins, 1982; Chen et al., 1982; Jarrett et al., 1984).

Entretanto, estudos realizados a partir do início da década atual, com o objetivo de investigar a real diversidade do BPV, têm indicado a existência de numerosos tipos de BPV, a exemplo do que ocorre nos seres humanos. O primeiro trabalho realizado envolveu o emprego

dos *primers* genéricos FAP em amostras (*swabs)* provenientes de pele saudável de animais pertencentes a 19 espécies de vertebrados. Dentre os dez bovinos analisados, e que não apresentavam qualquer sinal clínico compatível com a infecção pelo BPV, em seis foram detectados um ou dois prováveis novos tipos de BPV. Estes prováveis novos tipos virais foram denominados BAA-1 a BAA-5 (Antonsson e Hansson, 2002).

Posteriormente, visando à determinação da prevalência do BPV em papilomas e pele saudável de tetos, um estudo que envolveu a utilização dos *primers* FAP59/FAP64 e MY09/MY11, analisou 15 papilomas de teto e 122 *swabs* de pele saudável de animais, provenientes de cinco municípios do Japão. Quatro tipos previamente caracterizados de BPV (BPV-1, -3, -5 e -6), dois prováveis novos tipos de BPV anteriormente identificados (BAA-1 e -5) e 11 prováveis novos tipos (designados BAPV-1 a -10 e BAPV-11MY) foram encontrados nas 39 amostras positivas para o BPV. Porém, os prováveis novos tipos BAA-1 e BAPV-7 a -10 foram somente detectados a partir de amostras obtidas de pele saudável (Ogawa et al., 2004). Adicionalmente, em um surto de papilomatose mamária (tetos), ocorrido no Japão em um rebanho constituído por 560 novilhas, foi confirmada a presença do BPV-6 na maioria das 14 amostras analisadas. Nessa amostragem também foram identificados os prováveis novos tipos BAA-5 e BAPV-1, anteriormente descritos (Maeda et al., 2007).

Recentemente, a realização de análises complementares de alguns prováveis novos tipos de BPV, por meio da determinação da seqüência completa do genoma viral, permitiu a caracterização de novos tipos virais. O primeiro novo tipo caracterizado foi o BPV-7, inicialmente denominado BAPV-6. Como a seqüência de nucleotídeos da ORF L1 do BPV-7 foi mais relacionada àquela de PVs membros dos gêneros *Betapapillomavirus*, *Gamapapillomavirus* e *Pipapillomavirus*, os quais contêm representantes responsáveis por lesões cutâneas em humanos e em mucosa de hamsters, este novo tipo de BPV, provavelmente, constituirá um novo gênero ainda não nominado (Ogawa et al., 2007).

O segundo tipo de BPV recentemente descrito foi o BPV-8, anteriormente designado como BAPV-2 e identificado no Japão. A definição deste novo tipo viral foi realizada juntamente com a descrição da sua variante denominada BPV-8-EB, proveniente de um caso de papilomatose cutânea em um bisão europeu nascido na Itália. A alta similaridade observada entre as seqüências da ORF L1 do BPV-8 e do BPV-5 (75%), assim como a análise filogenética, constituíram a base para a classificação deste novo tipo viral no gênero *Epsilonpapillomavirus*. Adicionalmente, a estrutura genômica das regiões inicial e tardia destes dois diferentes membros do gênero se mostrou quase idêntica, diferindo apenas na ORF E4, que está presente no BPV-8 e ausente no BPV-5 (Tomita et al., 2007).

Os dois tipos de BPV descritos mais recentemente, identificados a partir de papilomas de tetos, foram o BPV-9 e o BPV-10, inicialmente denominados prováveis novos tipos BAPV-1 e BAA-5. A análise filogenética e a constatação da maior similaridade na ORF L1 com o BPV-3 (74,2 e 71,2%, respectivamente) classificaram os dois novos isolados no gênero *Xipapillomavirus* (Hatama et al., 2008).

No Brasil, a associação entre a infecção pelo BPV e a ocorrência de papilomatose cutânea, hematúria enzoótica e neoplasias do trato digestório superior, tem sido confirmada em bovinos. Estudos anteriormente realizados, empregando *primers* específicos para um único tipo viral, demonstraram a presença do BPV-1 em lesões cutâneas, sangue total e plasma sangüíneo de animais adultos com papilomatose cutânea, e em placenta e líquido amniótico provenientes de uma vaca apresentando o mesmo quadro clínico. O BPV-2 foi identificado em sangue periférico e bexiga urinária de animais com hematúria enzoótica, e em amostras de papilomas e do trato reprodutivo feminino de bovinos (Dos Santos et al. 1998; De Freitas et al., 2003; De Carvalho et al., 2003; Wosiacki et al., 2005, 2006).

Apesar da papilomatose cutânea representar um problema sanitário considerável, tanto para rebanhos bovinos de corte quanto, principalmente, leiteiros, a realização de estudos

visando à identificação dos tipos de BPV envolvidos na determinação de lesões cutâneas em bovinos no Brasil ainda é esporádica. Recentemente, a detecção do BPV-1, -2, -6 e -8 em papilomas provenientes de rebanhos bovinos do Estado do Paraná foi possível por meio da utilização dos *primers* genéricos FAP (Claus et al., 2007; 2008a, *in press*). Em outro estudo, a identificação de quatro prováveis novos tipos de BPV, denominados BPV/BR-UEL2 a -5, ainda não descritos no mundo, aponta para a ocorrência de considerável diversidade viral nos rebanhos bovinos brasileiros (Claus et al., 2008b, *in press*).

Atualmente, tem-se grande expectativa de que o delineamento de estudos mais abrangentes e focados na epidemiologia molecular das infecções pelo BPV possam evidenciar a ocorrência de grande diversidade viral, assim como ocorre em HPV. Com a ratificação dessa hipótese, será possível afirmar que os numerosos tipos de BPV passaram indetectáveis ao longo dos anos não devido à inexistência de variabilidade viral, mas sim porque técnicas inadequadas, inclusive de biologia molecular, levaram a sua não detecção.

Como a imunidade humoral e a celular induzida pelo BPV é tipo-específica, a definição dos tipos virais envolvidos em lesões neoplásicas em bovinos não tem apenas interesse epidemiológico. Com a definição dos tipos de BPV mais freqüentes em quadros clínicos que, devido à sua freqüência de ocorrência, ocasionam prejuízos econômicos à exploração pecuária, será possível definir os tipos virais que poderiam representar alvos para o desenvolvimento de vacinas tipo-específicas.

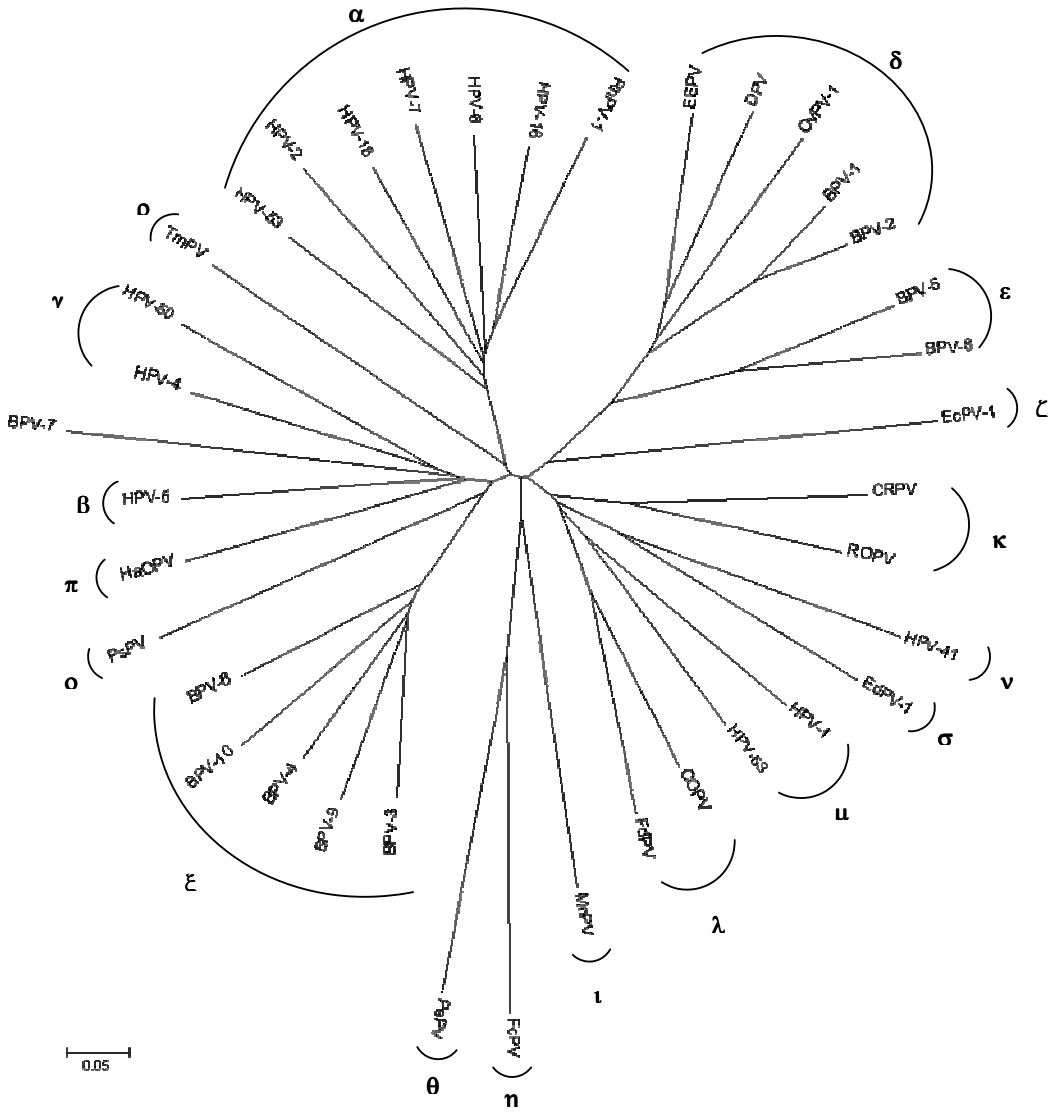

Fig. 1. Reconstrução filogenética baseada em seqüências completas do gene L1 de 39 papilomavírus humanos e animais, classificados nos gêneros *Alphapapillomavirus* ao *Sigmapapillomavirus*.

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2. OBJETIVOS

2. OBJETIVOS

2.1. Objetivo geral

- Determinar o posicionamento filogenético (gênero) de um provável novo tipo de BPV (BPV/BR-UEL2) por meio da caracterização molecular da ORF L1.

2.2. Objetivos específicos

- Desenvolver e avaliar uma PCR com a utilização de *primers* genéricos desenhados a partir de seqüências dos genes L2, L1 e da região LCR de tipos de BPV pertencentes ao gênero *Xipapillomavirus* (BPV-3, -4 e -6);

- Clonar os *amplicons* obtidos na PCR e seqüenciar os plasmídeos extraídos dos clones gerados;

- Obter uma seqüência consensual representativa da ORF L1 da amostra BPV/BR-UEL2;

- Determinar o percentual de similaridade da ORF L1 do provável novo tipo viral BPV/BR-UEL2 com aquelas referentes aos 10 tipos de BPV disponíveis em bases públicas de dados;

3. ARTIGO PARA PUBLICAÇÃO

PHYLOGENETIC POSITION OF A BRAZILIAN UNCHARACTERIZED BOVINE PAPILLOMAVIRUS TYPE IN THE *XIPAPILLOMAVIRUS* GENUS BY SEQUENCING OF L1 OPEN READING FRAME

Artigo editado de acordo com as normas de publicação do periódico

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Phylogenetic position of a Brazilian uncharacterized bovine papillomavirus type in the *Xipapillomavirus* genus by sequencing of L1 open reading frame

Abstract

In Brazil, despite the endemic characteristic of BPV infections, the identification of BPV types in cattle is still sporadic. However, in a recent investigation, through the analysis of a partial segment of L1 gene, we could verify a notable diversity among the BPV types detected in papilloma specimens from four cattle herds. The aim of the current study is to state the phylogenetic position of a previously identified putative new BPV type (BPV/BR-UEL2) detected in Parana state, Brazil. The BPV/BR-UEL2 was isolated from a papilloma located in the axillary region of a dairy cow. As the previous FAP sequence analysis had revealed our isolate as closest related to BPV type 4, alignments of L1, L2, LCR genomic regions of some *Xipapillomavirus* representatives (BPV-3, -4, and -6) were used in design of degenerate primers. In addition, the previously described FAP primer pair was also employed in both the original form as in combination with the designed primers. The three overlapping amplicons obtained from the L1 ORF, with 435, 475, and 1128 bp, were submitted to cloning and then sequenced. Phylogenetic analysis with complete L1 ORF sequences revealed the BPV/BR-UEL2 isolate as related with BPV types held in *Xipapillomavirus* genus, displaying the highest L1 nucleotide sequence similarity with BPV type 4 (78%), what suggests its classification in the *Xipapillomavirus* genus.

Key words: Bovine; Cutaneous papillomatosis; Bovine papillomavirus; Putative new BPV type; L1 gene; Phylogenetic analysis.

Introduction

Papillomaviruses (PVs) are a highly diverse group of circular double-stranded DNA viruses that can induce epithelial proliferations in a wide range of vertebrate species. In cattle, the bovine papillomavirus (BPV) has been implied as the casual agent of cutaneous papillomatosis, and cancer of urinary bladder and upper gastrointestinal tract (Campo, 2002).

PVs have a similar genomic organization, with all viral genes located on one DNA strand. The coding strand can present up to ten open reading frames (ORFs) that are classified as either early (E) or late (L) ORFs, according to their position in the genome. The early region of genome is constituted by up to eight ORFs, which encodes the viral regulatory proteins. The late region usually encodes two viral structural proteins (L1 and L2), which give rise to the viral capsid. Additionally, there is a non-coding region in genome which has been designated as long control region (LCR) (Howley and Lowy, 2001).

Recently, based on comparison of entire L1 nucleotide sequence of almost all known PVs, it was defined that the family *Papillomaviridae* is formed by 18 genera (*Alphapapillomavirus* to *Sigmapapillomavirus*), being each one composed of a varied number of species. Besides, it was established that different genera display less than 60% identity in the L1 nucleotide sequence, while diverse species within a genus share between 60 and 70% identity. However, the traditional types within a species present between 71 and 89% identity in the same gene (De Villiers et al., 2004).

While more than a hundred of human papillomavirus (HPV) types have been characterized, there were only six BPV types described in cattle before 2007 (Pfister et al., 1979; Campo et al., 1980, 1981; Campo and Coggins, 1982; Chen et al., 1982; Jarrett et al., 1984). These BPVs were classified in the *Deltapapillomavirus* (BPV-1 and -2), *Xipapillomavirus* (BPV-3, -4, and -6), and *Epsilonpapillomavirus* (BPV-5) genera (De Villiers et al., 2004). In addition, the most recently characterized BPV types were held in

Epsilonpapillomavirus (BPV-8) and *Xipapillomavirus* (BPV-9 and -10), with the exception of BPV-7 which was known to belong to a yet unassigned PV genus (Tomita et al., 2007; Ogawa et al., 2007; Hatama et al., 2008).

As observed in HPV, the use of PCR assay with degenerate primers, which amplify partial fragments of L1 gene, followed by sequencing, have demonstrated the occurrence of numerous BPV types throughout cattle herds worldwide. By using the FAP59/FAP64 and MY09/MY11 primers, it was possible the detection of twelve putative new BPV types in both teat skin warts and healthy teat skin of cattle from Japan and Sweden (Manos et al., 1989; Forslund et al., 1999; Antonsson and Hansson, 2002; Ogawa et al., 2004; Maeda et al., 2007).

In Brazil, a recent investigation employing the same methodology has revealed a notable diversity among the BPV types detected in papillomas of four different cattle herds from Paraná state. Moreover, this study has demonstrated the identification of four putative new BPV types, denominated as BPV/BR-UEL2 to 5 (GenBank Accession Nos. EU293538 to EU293541, respectively) (Claus et al., 2008a, *in press*).

The aim of the current study is to state the phylogenetic position of a previously identified putative new BPV type (BPV/BR-UEL2) detected in Brazil.

Materials and Methods

Papilloma specimen and cell lysis

The BPV/BR-UEL2 DNA was isolated from a rice-grain papilloma located in the axillary region of a cow belonging to a dairy cattle herd from the Parana state, the southern region of Brazil (Claus et al., 2008a, *in press*). The papilloma specimen was taken by hand (wearing gloves) and a fragment was triturated in phosphate buffered saline (PBS pH 7.2). The suspension (10% w/v) was centrifuged for 15 min at 1500 x g at 4°C. An aliquot (250 µL) of the supernatant was treated with lysis buffer [10mM Tris; 1mM EDTA; 0.5% Nonidet

P40; 1% SDS; and 0.2 mg/mL proteinase K (Invitrogen, Life Technologies, USA)]. After homogenization, the sample was incubated at 56°C for 30 min.

DNA extraction

For DNA extraction, a combination of phenol/chloroform/isoamyl alcohol and silica/guanidine isothiocyanate methods was performed (Alfieri et al., 2006). Briefly, the supernatant was treated with an equal volume (500 µL) of phenol/chloroform/isoamyl alcohol (25:24:1), homogenized and heated at 56°C for 15 min (Sambrook and Russell, 2001). After centrifugation at 10,000 x *g* for 10 min, the aqueous phase was mixed with silica/guanidine isothiocyanate (Boom et al., 1990). The DNA was eluted in 50 µL of ultrapure (MilliQ®) sterile water and kept at −20°C until use. An aliquot of ultrapure sterile water was included as negative control in the DNA extraction procedure.

Strategy and primer design

To achieve the entire L1 nucleotide sequence of the BPV/BR-UEL2 isolate, the following strategy was used. As the previous FAP sequence analysis of 475 nt (GenBank Accession No. EU293538) had revealed the BPV/BR-UEL2 isolate as closest (77%) related to BPV type 4, alignments of amino acid sequences from L2, and nucleotide sequences from L1 and LCR regions of some *Xipapillomavirus* representatives (BPV-3, -4, and -6), were used in design of degenerate primers.

Aiming for amplification of the upstream region of L1 ORF, which includes the start codon, a first primer set was designed. The forward primer (L2Bf) was obtained from a terminal conserved region of L2 sequence, and the reverse (L1Br) from the FAP amplicon sequence previously determined for the BPV/BR-UEL2.

To reach the following segment, which comprises almost the entirety of the first half of L1 gene, the previously described FAP primer pair was employed (Forslund et al., 1999).

A third primer pair was designed to amplify the terminal half of L1 ORF. The forward primer (L1Bf) was also obtained from FAP amplicon sequence of our isolate. However, the reverse primer (LCRBr) was reached from a relatively conserved upstream region verified in LCR sequences of BPV types 3, 4, and 6.

When the designed primer pairs were not able to amplify the desired segments, combinations between them and the FAP primer pair were evaluated (see table 1 for primers features). In addition, the same primer sets were also evaluated on DNA samples previously known to presence of *Xipapillomavirus* representative BPV type 6.

For alignment of sequences and primer design, the CLUSTAL W Multiple Alignment program and Gene Runner version 3.05 program (Hastings Software Inc., Hastings, NY), were respectively used (Thompson et al., 1994).

PCR parameters

The PCR reactions contained 2.5 µL of DNA sample, 0.5 µL (20 pmol) of each primer, 200 µM of each dNTP, 2.5 U of *Platinum Taq* DNA polymerase (Invitrogen Life Technologies, USA), 1x PCR buffer (20 mM Tris-HCl pH 8.4 and 50 mM KCl), 1.5 mM of magnesium chloride and ultrapure sterile water to a final volume of 25 µL. PCR reactions were carried out in a thermocycler PTC-200 (MJ Research Co., USA), using the following time and temperature conditions: 10 min at 94°C, and then 40 cycles of 1 min at 94°C, 1 min at an optimum temperature for annealing, 1 min at 72°C, and a final extension of 10 min at 72°C. The annealing temperatures for primer pairs L2Bf/FAP64, L2Bf/L1Br, and L1Bf/LCRBr, were 50, 54 and 57°C, respectively.

The amplified products were analyzed by electrophoresis in 2% agarose gel in TBE buffer pH 8.4 (89 mM of Tris; 89 mM of boric acid; 2 mM of EDTA) at constant voltage (90V) for approximately 45 min, stained with ethidium bromide (0.5 μg/mL), and visualized under UV light.

Cloning and DNA sequencing

Initially, all PCR products were purified using PureLink Quick Gel Extraction Kit (Invitrogen, Life Technologies, USA), and then the cloning using the TOPO TA Cloning kit for Sequencing (Invitrogen, Life Technologies, USA) was carried out according to the manufacturer's instructions.

After that, the sequencing of plasmid DNA from two selected clones, for each PCR amplicon, was performed by using the DYEnamic ET dye terminator cycle sequencing kit (Amersham Biosciences, UK) with M13 forward and reverse primers, in a MegaBACE 1000/Automated 96 Capillary DNA Sequencer (GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions.

Sequence analysis

The obtained sequences were examined with the PHRED software (http://bioinformatica.ucb.br/electro.html) for quality analysis of chromatogram readings. The sequences were accepted if base quality was equal to or higher than 20. The consensus sequence was determined using the CAP3 software (http://bioinformatics.iastate.edu/aat/sas.htmL) and the sequence identity was verified with all sequences deposited in the GenBank using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST).

The L1 ORF of the Brazilian isolate was predicted by analysis with the ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.htmL). The alignment and degree of similarity among sequences at both nucleotide and amino acid levels were determined using BIOEDIT version 5.0.9 software (Hall, 1999). The phylogenetic tree was obtained by the Neighbour-joining method with the Kimura two-parameter distance estimate (Kimura, 1980), using MEGA version 3.1 program (Kumar et al., 2004). Bootstrap support values were determined for 1000 replications.

Results

The first L1 segment of the Brazilian isolate could be achieved by a semi-nested PCR system (SN-PCR) employing L2Bf/FAP64 primers in the first round, and L2Bf/L1Br primer pair in the second round, which yielded an amplicon of 435 bp. The use of the FAP59/FAP64 (475 bp) and L1Bf/LCRBr (1128 bp) primer sets allowed the amplification of the remaining portions of the same gene (figure 1).

A consensual sequence of 1804 nt, that spans from nt 5385 to 7184 of BPV-4, was achieved from L2Bf/L1Br, FAP59/FAP64 and L1Bf/LCRBr overlapping sequences of the BPV/BR-UEL2 isolate.

Phylogenetic analysis employing complete L1 ORF sequences revealed that the BPV/BR-UEL2 isolate is related with BPV types held in *Xipapillomavirus* genus (Figure 2). Besides, by pairwise DNA sequence alignment, this isolate displayed the highest L1 nucleotide sequence similarity with BPV type 4 (78%), suggesting its classification in the *Xipapillomavirus* genus. On the other hand, when the comparison was determined at the amino acid level, the percentage identity with the BPV-4 was 83,6%.

In regard to amplification of DNA samples previously known to BPV-6 presence, the three overlapping amplicons could be obtained in all samples tested.

Discussion

In the current study, we have stated the phylogenetic position of a Brazilian potential novel BPV type, the isolate BPV/BR-UEL2, through the determination of its entire L1 nucleotide sequence by a PCR assay employing degenerate primers.

Despite several attempts in reaching a distinct band by employing the L2Bf/L1Br primer pair at diverse annealing temperatures, only weak bands with background amplification could be obtained to the BPV/BR-UEL2 isolate. Therefore, the upstream region of its L1 ORF was obtained by a SN-PCR whose first round was performed with L2Bf/FAP64 primers, while the second employed the L2Bf/L1Br primer pair. On L1Bf/LCRBr PCR reaction, the expected amplicon could be achieved, with great intensity, by using annealing temperatures ranging from 50 to 57°C.

As demonstrated by both the pairwise DNA sequence alignment and the phylogenetic analysis with other BPV types whose complete L1 sequences are available, the referred isolate was closest related with the BPV-4, being classified in *Xipapillomavirus* genus, in which are also found BPV-3, -6, and the most recently described types, BPV-9 and -10. The L1 nucleotide sequence of our isolate presented identities with the *Xipapillomavirus* representatives ranging from 71 to 78%, while similarities with other genera representatives ranged from 55,6 to 55,9%. A phylogenetic analysis based on amino acid alignments was also done and the generated tree presented a topology very similar to that based on nucleic acid alignment (data not shown).

When compared with other BPV genera, the *Xipapillomavirus* genus has demonstrated a promising great diversity in BPV types. Such fact has been pointed by diverse studies in which several putative new BPV types have been comprised together with representatives of this genus; e.g. BAA-1, BAPV-3, -8, -9 and -10, and BPV/BR-UEL3 (Antonsson and Hansson, 2002; Ogawa et al., 2004; Claus et al., 2008a, *in press*).

The BPV type 4 has been associated with tumors of the alimentary canal of cattle (Campo et al., 1980, 1994; Borzacchiello et al., 2003). Differently, our Brazilian isolate was detected from a skin wart located in the axillary region of a dairy cow, and then was associated with cutaneous papillomatosis.

Despite the closest similarity with BPV-4, the L1 ORF encoded protein of this Brazilian isolate consisted of 532 amino acids, such as L1 protein of BPV-9, a new described *Xipapillomavirus* type, which consisted of 531 amino acids. Otherwise, the BPV-4 L1 ORF codes for a protein of 506 amino acids. Additionally, the ORF analysis of the obtained consensual sequence demonstrated an overlapping pattern between L2 and L1, which could also be verified in BPV-9 and -10, and differed from observed in BPV-4.

To evaluate the ability of the designed primers on amplifying the expected segments of other *Xipapillomavirus* representative, the same PCR strategy was applied to some DNA samples from skin warts previously known to presence of BPV type 6. Differently from our putative novel BPV type, the upstream fragment of L1 gene of BPV-6 could be achieved by PCR employing the L2Bf/FAP64 primer set, whereas the subsequent L1 segments were obtained by using the same primer pairs as performed to BPV/BR-UEL2 isolate (data not shown). The specificity of the amplified products was confirmed for one of these samples through the analysis of the resulting consensual sequence.

PCR assays with degenerate primers displaying a high degree of nucleotide identity with conserved regions in L1 gene, and primarily aimed for detection of cutaneous or genital

HPV types, have been satisfactorily applied for the detection of a broad range of PV types in both humans and other animal hosts. Some advantages presented by such methods have been the detection of unreported PV types, the possibility of performing reliable studies of PV prevalence, in skin lesions and normal skin, and a rapid identification of the PV type through a single PCR reaction (Manos et al., 1989; Forslund et al., 1999).

The strategy developed in this study involved the use of three pair of degenerate primers, being two of them specifically designed for amplification of L1 gene of some *Xipapillomavirus* representatives. It has showed of great value once it allowed the achieving of entire L1 gene sequence, unambiguously and easily, and thus the determination of the correct phylogenetic position of an uncharacterized BPV type.

The use of simple and rapid methods to obtain the whole L1 gene sequence, such as the strategy designed and applied in this study, is of fundamental importance not only for stating the genetic classification. The ready availability of entire L1 nucleotide sequences, from all putatively new BPV types detected so far, could allow an improvement of the current PCR assays. Since the degenerate primers which have been employed were primarily designed to amplify only HPV sequences, an adequacy of such primers to L1 sequences from BPV types would be necessary. Given the possibility to increase the detection of novel BPV types by an optimized degenerate primer PCR approach, the actual genotype diversity for the bovine species could extend substantially.

In Brazil, despite the relatively common occurrence of BPV infections, the identification of BPV types in cattle herds is still sporadic. Besides, the few studies that have been done usually involve the use of type-specific primers and determined the presence of BPV types 1 and 2 (Dos Santos et al., 1998; De Freitas et al., 2003; Wosiacki et al., 2005, 2006). Recently, the use of the FAP59/FAP64 primer pair enabled the identification both of previously described BPV types (BPV-1, -2, -6, and -8) and of four putative new BPV types,

not yet described around the world, from skin warts of cattle from Parana state (Claus et al., 2007; 2008a, *in press*; 2008b, *in press*).

In the present study, we determine the phylogenetic position of an uncharacterized BPV type detected from a cutaneous lesion of a dairy cow. The realization of further studies involving the molecular epidemiology of BPV infections, in Brazilian cattle herds as much in diverse geographical areas around the world, could indicate its prevalence throughout the cattle as well as check its association with cutaneous lesions. In the most recent example, the BPV-8, the first detection was made from papillomas and healthy skin of teats from Japanese cattle herds, and subsequent investigations have revealed its presence in papilloma specimens from a European bison kept in Slovakia and a Brazilian cow (Literák et al., 2006; Tomita et al., 2007; Claus et al., 2008b, *in press*).

Due to the fact that there are so few studies involving BPV molecular characterization, one might speculate that the BPV/BR-UEL2 is spread in susceptible herds from many regions, especially in Brazil.

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Table 1. Sequence and features of polymerase chain reaction primers.

Primer	Genomic region targeted	Polarity	Sequence	Nucleotide positions[a]	Degeneracy degree
L2Bf	L2	+	5'GTIAARYTITTYATHAAYGAYGC3'	5385-5407	96
FAP59[b]	L1	+	5'TAACWGTIGGICAYCCWTATT3'	5729-5749	8
L1Br	L1	–	5'AASACTCTGAATTGACTGCC3'	5794-5813	2
L1Bf	L1	+	5'GRGAGCAYTGGGAYAAAG3'	6089-6106	8
FAP64[b]	L1	–	5'CCWATATCWVHCATITCICCATC3'	6175-6197	36
LCRBr	LCR	–	5'CWRCATTTTATTKSSAASATTC3'	7181-7202	64

[a] Relative position in BPV-4 genome.

[b] Forslund et al. (1999).

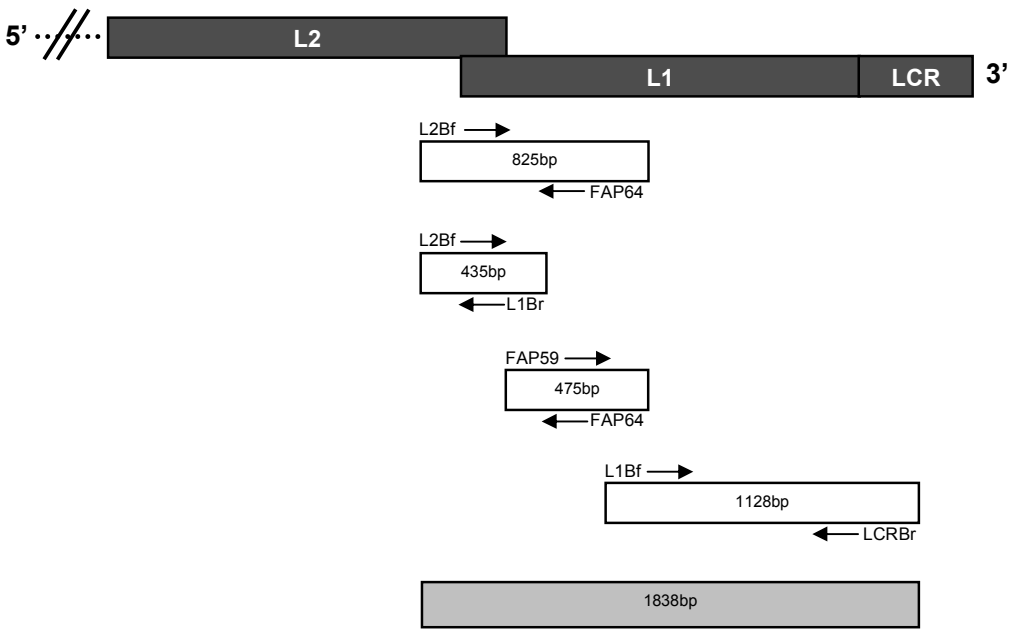

Fig. 1. Schematic diagram showing the relative positions of the overlapping PCR fragments in L2, L1 and LCR regions of BPV/BR-UEL2 isolate. The length of each amplicon is indicated into the white box, while the primer sets are shown as arrows. The grey box represents the consensual sequence obtained.

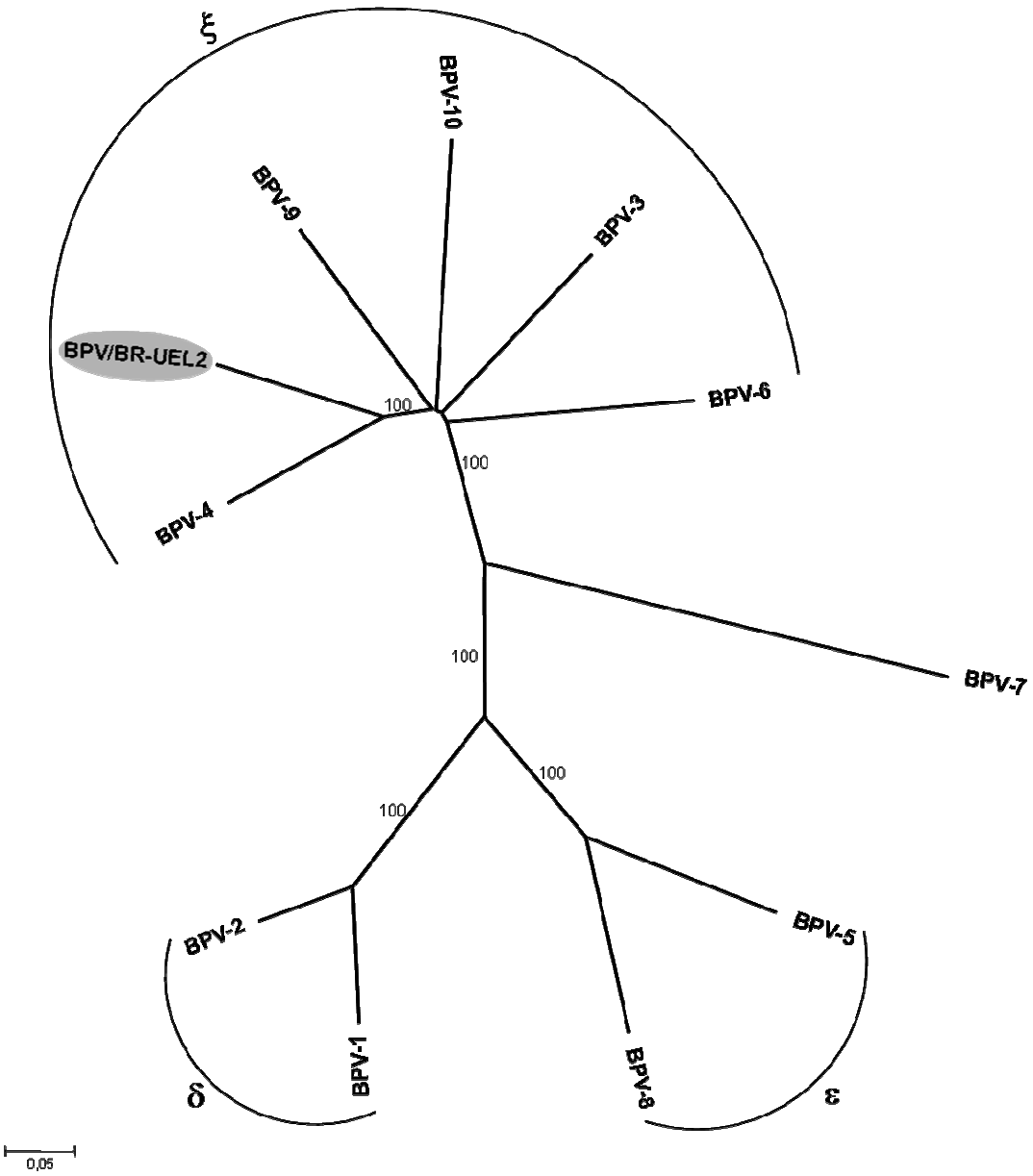

Fig. 2. Neighbour-joining phylogenetic tree of L1 ORFs (nt) of the BPVs, including that of the BPV/BR-UEL2 isolate (indicated by shading). The tree is divided into the previously determined genera *Deltapapillomavirus* (BPV-1 and -2), *Epsilonpapillomavirus* (BPV-5 e -8), *Xipapillomavirus* (BPV-3, -4, -6, -9, -10) and an unassigned PV genus (BPV-7). The numbers at the internal nodes represent the bootstrap support values determined for 1000 replications.

4. CONCLUSÕES

4. CONCLUSÕES

- O sistema de PCR desenvolvido neste trabalho permitiu a amplificação da totalidade do gene que codifica a proteína estrutural L1 de um provável novo tipo de BPV (BPV/BR-UEL2);

- A seqüência consensual representativa da ORF L1 da amostra BPV/BR-UEL2, obtida a partir de clones, e a sua comparação com seqüências do mesmo gene de outros tipos de BPV descritos anteriormente, permitiu a classificação deste provável novo tipo de BPV no gênero *Xipapillomavirus*;

- A aplicabilidade deste sistema, envolvendo o uso de *primers* genéricos, em outro representante do gênero *Xipapillomavirus* (BPV tipo 6) pôde ser confirmada pela obtenção dos *amplicons* correspondentes em amostras previamente caracterizadas como BPV-6, e pela confirmação da especificidade dos mesmos por meio de clonagem, seqüenciamento e análise filogenética.

APÊNDICES

APÊNDICE A: Lista de Reagentes

1. 100 mM dNTP Set, 4 x 250 µL; 25 µmol each (100 mM dATP Solution, 100 mM dCTP Solution, 100 mM dGTP Solution, 100 mM dTTP Solution) (Invitrogen Life Technologies®)
2. 10 x PCR-Buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl) (Invitrogen Life Technologies®)
3. 123 bp DNA Ladder (Invitrogen Life Technologies®)
4. 2-Mercapto-ethanol ($C_2H_6O_5$) P.M. 78,13 (Fluka®)
5. Acetona, P.A. (CH_3COCH_3) P.M. 58,08 (Dinâmica®)
6. Ácido acético glacial, P.A. (CH_3COOH) P.M. 60,05 (Nuclear®)
7. Ácido bórico (H_3BO_3) P.M. 61,83
8. Ácido clorídrico (HCl) P.M. 36,46 (Reagen®)
9. Ácido etilenodiaminotetraácido Sal di-sódico – EDTA, P.A. ($C_{10}H_{14}N_2O_8Na_22H_2O$) P.M. 372,24 (Reagen®)
10. Agarose (Gibco BRL®)
11. Ágar (Himedia Laboratories®)
12. Álcool etílico absoluto (C_2H_2OH) P.M. 46,07 (Nuclear®)
13. Álcool isoamílico ($(CH_3)_2CHCH_2CH_2OH$) P.M. 88,15 (Synth®)
14. Ampicilina trihidratada (USB®)
15. Azul de bromofenol (Sigma®)
16. Cloreto de Potássio, P.A. (KCl) P.M. 74,56 (Reagen®)
17. Cloreto de Sódio, P.A. (NaCl) P.M. 58,45 (Reagen®)
18. Clorofórmio, P.A. ($CHCl_3$) P.M. 119,38 (Dinâmica®)
19. Dodecil Sulfato de Sódio – Lauril Sulfato de Sódio – SDS ($C_{12}H_{25}NaO_4S$) P.M. 288,38 (Synth®)
20. Ethidium bromide ($C_{21}H_{20}N_3Br$) P.M. 394,3 (Sigma®)
21. Extrato de Levedura (USB®)
22. Gibco BRL – Concert™ Rapid Plasmid purification System (Invitrogen Life Technologies®)
23. Glicina, P.A. (Nuclear®)
24. Guanidine isothiocyanate P.M. 118,16 (Gibco BRL®)
25. Hidróxido de Sódio, P.A. (NaOH) P.M. 40,00 (Dinâmica®)

26. Hidroximetil amino metano – TRIS 99% P.M. 121,14 (Inlab®)
27. Lambda DNA - (Invitrogen Life Technologies®)
28. Metanol, P.A. (CH_3OH) P.M. 32,04 (Allkimia®)
29. *Platinum* Taq DNA Polymerase recombinant 500 units (Invitrogen Life Technologies®)
30. PureLink Quick Gel Extraction Kit (Invitrogen Life Technologies®)
31. Sacarose, P.A. – sucrose ($C_{12}H_{22}O_{11}$) P.M. 342,31 (Reagen®)
32. Silicon dioxide (SiO_2) P.M. 60,08 (Sigma®)
33. TOPO TA Cloning kit for Sequencing (Invitrogen Life Technologies®)
34. Triton x-100
35. Triptona (Acumedia Manufacturers®)

APÊNDICE B: Soluções e Tampões

- **Hidratação da sílica**
  - 60 g de sílica (SIGMA®)
  - Adicionar 500 mL de água MilliQ autoclavada
  - Agitar lentamente e manter em repouso durante 24 hs
  - Por sucção, desprezar 430 mL do sobrenadante
  - Ressuspender a sílica em 500 mL de água bidestilada
  - Manter em repouso durante 5 hs para sedimentar
  - Desprezar 440 mL do sobrenadante
  - Adicionar 600 μL de HCl para ajustar o pH (pH=2,0)
  - Aliquotar e autoclavar

- **Solução L6**
  - 120 g de isotiocianato de guanidina (GUSCN)
  - 100 mL de TRIS-HCl 0,1 M pH 6,4
  - 22 mL de EDTA 0,2 M pH 8,0
  - 2,6 mL de Triton x-100

- **Solução L2**
  - 120 g de tiocianato de guanidina (GUSCN)
  - 100 mL de TRIS-HCl 0,1 M pH 6,4

- **Tampão de Amostra**
  - Azul de bromofenol 0,25%
  - Sacarose – sucrose ($C_{12}H_{22}O_{11}$) 45%

- **Tampão de corrida: TBE (TRIS – Ácido bórico – EDTA) 10 x [ ]**
  - 0,89 M TRIS
  - 0,89 M ácido bórico
  - 0,02 M EDTA dissodium
  - Água bidestilada qsp. 1 litro

    pH = 8,4

- **Tampão fosfato salina (PBS)**

  -137 mM cloreto de sódio (NaCl)

  - 3 mM cloreto de potássio (KCl)

  - 8 mM sódio fosfato dibásico anidro (Na_2HPO_4)

  - 15 mM potássio fosfato monobásico ($K_2H_2PO_{4)}$

  - Água MilliQ autoclavada q.s.p. 1 litro

    pH = 7,2

- **Fenol / clorofórmio / álcool isoamílico (24:24:1)**

  - 24 mL fenol saturado

  - 24 mL clorofórmio

  - 1 mL álcool isoamílico

- **Meio Luria Bertani (LB) sólido para 500 mL**

  - 5 g de triptona

  - 2,5 g de extrato de levedura

  - 2,5 g de NaCl

  - Dissolver em 400 mL de água destilada

  - Completar para 500 mL (becker).

  - Pesar 3,25 g de ágar em cada erlenmeyer.

  - Adicionar 250 mL da solução a 3,25g de ágar em cada erlenmeyer que será autoclavado.

- **Meio LB líquido**

  - 5 g de triptona

  - 2,5 g de extrato de levedura

  - 2,5 g de NaCl

  - Dissolver em 400 mL de água destilada e completar para 500 mL.

  - Aliquotar a solução em tubos de 3 mL e 5 mL e autoclavar.

APÊNDICE C: Protocolo de Técnicas

- **Suspensão de Fragmentos de Tecidos**

  - Triturar 1 g do fragmento do tecido (papiloma) com pistilo em gral de porcelana estéril
  - Adicionar 9 mL de PBS 1x
  - Homogeneizar em vórtex, congelar, descongelar rapidamente por 3 vezes
  - Centrifugar a 4.000 x g /15 min
  - Recolher 500 μL do sobrenadante em microtubos tipo eppendorf para extração do DNA

- **Lise Celular**

  - 250 μL do sobrenadante do macerado
  - Diluir em 250 μL de PBS 1x
  - 50 μL de SDS 10 %
  - 10 μL de proteinase K (0,2 mg/mL)
  - Homogeneizar em vórtex por 10 s
  - Incubar em banho-maria à 56^{o}C / 30 min

- **Extração do DNA pela técnica fenol / clorofórmio / álcool isoamílico - sílica / isotiocianato de guanidina**

  - Adicionar 500 μL de fenol / clorofórmio / álcool isoamílico, após a lise celular
  - Homogeneizar em vórtex
  - Incubar em banho-maria à 56^{o}C / 15 min
  - Homogeneizar em vórtex
  - Centrifugar a 10.000 x g /12 min
  - Recolher a fase aquosa em outro microtubo tipo eppendorf
  - Adicionar 25 μL de sílica hidratada
  - Adicionar 1.000 μL de solução L6
  - Homogeneizar em vórtex
  - Agitar durante 30 min em temperatura ambiente
  - Centrifugar a 10.000 x g / 30 s
  - Desprezar o sobrenadante em solução contendo NaOH 10 M
  - Adicionar 500 μL de solução L2
  - Homogeneizar em vórtex

- Centrifugar a 10.000 x g / 30 s
- Desprezar o sobrenadante em solução contendo NaOH 10M
- Adicionar 500 µL de solução L2
- Homogeneizar em vórtex
- Centrifugar a 10.000 x g / 30 s
- Desprezar o sobrenadante em solução contendo NaOH 10M
- Adicionar 1.000 µL de etanol 70% a -20^{o}C
- Homogeneizar em vórtex
- Centrifugar a 10.000 x g / 30 s
- Desprezar o sobrenadante
- Adicionar 1.000 µL de etanol 70% a -20^{o}C
- Homogeneizar em vórtex
- Centrifugar a 10.000 x g / 30 s
- Desprezar o sobrenadante
- Adicionar 1.000 µL de acetona PA a -20^{o}C
- Homogeneizar em vórtex
- Centrifugar a 10.000 x g / 2 min
- Desprezar o sobrenadante
- Secar o pellet em banho-maria a 56^{o}C / 15 min
- Adicionar 50 µL de água milliQ autoclavada
- Homogeneizar em vórtex
- Incubar em banho-maria à 56^{o}C / 15 min
- Homogeneizar em vórtex
- Centrifugar a 10.000 x g / 2 min
- Recolher o sobrenadante
- Estocar a –20^{o}C

- **Gel de agarose a 2%**
  - 1 g de agarose
  - 50 mL TEB 1 x
  - 30 µL de brometo de etídio

- **Purificação de produto de PCR excisado do gel**

1. Pesar o fragmento excisado do gel (até 400 mg) em um tubo de microcentrífuga (1,5 mL).
2. Adicionar 30 μL do tampão GS1 para cada 10 mg de gel.
3. Incubar o tubo à 50°C / 15 min, homogeneizando a cada 3 min.
4. Após dissolver, incube por mais 5 min.
5. Colocar a coluna em um novo tubo e pipetar a solução anterior na coluna.
6. Centrifugar por 12.000 x g / 1 min.
7. Descartar o filtrado e recolocar a coluna no mesmo tubo.
8. Adicionar 700 μL de tampão W9 e incubar a temperatura ambiente / 5 min.
9. Centrifugar a 12.000 x g / 1 min.
10. Descartar o filtrado e recolocar a coluna.
11. Centrifugue a coluna a 12.000 x g / 1 min para remover o tampão residual.
12. Colocar a coluna em um novo tubo (1,5 mL).
13. Adicionar 20 μL de água a 65°C no centro da coluna.
14. Incubar a temperatura ambiente por 2 min.
15. Centrifugar a 12.000 x g / 2 min.

- **Ligação do produto da PCR ao vetor**

1. Em um tubo de microcentrífuga (0,6 mL) adicionar:

Solução do DNA a ser clonado	0,5 a 4 μL
Solução de sal	1,0 μL
H_2O estéril	6 μL q.s.p
Vetor TOPO	1,0 μL

2. Homogeneizar a reação delicadamente e incubar por 1h.
3. Manter a reação em gelo até proceder a transformação.

- **Pré-inoculação de *E.coli* One Shot®**

1. 3 mL de meio LB líquido autoclavado.
2. Acrescentar 15 μL de células para cada 3 mL de LB.
3. Homogeneizar lentamente o tubo com LB.

4. Incubar em *Shaker* à 37°C e 180 rpm, *overnight*.

- **Preparação de células competentes**

1. Diluir a pré-cultura (150 µL em 5 mL de meio LB líquido) e incubar à 37°C, 180 rpm, até DO_{600nm}=0,4-0,6 (aproximadamente 1h).
2. Logo após, deixar o tubo em banho de gelo por 5 min.
3. Centrifugar 3 mL da cultura em 2 tubos de microcentrífuga (1,5 mL em cada tubo), a 5000 rpm / 5 min.

OBS: Manter as células e soluções em banho de gelo.

4. Eliminar o sobrenadante por inversão do tubo e adicionar ao precipitado 500 µL de 50mM $CaCl_2$.
5. Homogeneizar.
6. Transferir o conteúdo dos 2 tubos para outro tubo.
7. Manter em banho de gelo por 10 min.
8. Centrifugar a 5000 rpm / 5 min e eliminar o sobrenadante posteriormente.
9. Ressuspender as células em 300 µL de 50mM $CaCl_2$.
10. Manter em banho de gelo por 20 min.
11. Aliquotar 50 µL de células competentes em tubos de microcentrífuga e estocar a -20°C.

- **Transformação**

1. Adicionar 3 µL do produto da ligação a 50 µL de células competentes e colocar em banho de gelo por 20 min.
2. Incubar a mistura por 2 min a 42°C em banho-maria.
3. Transferir imediatamente para banho de gelo.
4. Adicionar 1 mL de LB líquido e incubar por 1h a 37°C sob agitação (180 rpm).
5. Centrifugar a 5.000 rpm / 10 min.
6. Descartar o sobrenadante.
7. Ressuspender o sedimento em 100 µL de LB líquido.
8. Semear em duas placas com LB sólido, contendo 75 µg / mL de ampicilina, com o auxílio da alça de Drigalsk.
9. Incubar em estufa a 37°C por 24h.

- **Extração do plasmídeo**

1. Aliquotar 5 μL de meio LB líquido em um tubo de ensaio.
2. Adicionar ampicilina (100 μg /mL).
3. Com o auxílio de um palito, realizar a semeadura de uma colônia de bactérias no tubo.
4. Incubar sob agitação (180 rpm) a 37°C / 12 h.
5. Transferir 1,5 mL da cultura em um tubo de microcentrífuga e centrifugar a 5.000 rpm / 12 min. Descartar o sobrenadante. Repetir o procedimento.
6. Adicionar 210 μL de G1 para ressuspender as células.
7. Adicionar 210 μL de G2 para lisar as células e inverter gentilmente cinco vezes. Manter à temperatura ambiente por 5 min.
8. Adicionar 280 μL de G3 para neutralizar e inverter gentilmente cinco vezes. Centrifugar a 12.000 rpm / 10 min.
9. Transferir o sobrenadante para a coluna.
10. Adicionar 700 μL de G4 (tampão de lavagem com etanol) no centro da coluna.
11. Centrifugar a 12.000 rpm / 1 min (duas vezes).
12. Adicionar 75 μL de água no centro da coluna. Incubar a temperatura ambiente por 1 min.
13. Centrifugar a 12.000 rpm / 2 min.

ANEXOS

ANEXO A: Seqüência do gene L1 do isolado BPV/BR-UEL2

```
1    ATG ACA CAG CTA CTT TTT TTC TAC ATC CCA GTC TGC TCA GAA AAC ATA AAC ATA AAC ATT 60
1     M   T   Q   L   L   F   F   Y   I   P   V   C   S   E   N   I   N   I   N   I  20

61   GGT TTC TTT AAT GTT TTA CAG ATG TCA TTC TGG CTA CCA AAC TCA GCA AAG CTG TAT TTA 120
21    G   F   F   N   V   L   Q   M   S   F   W   L   P   N   S   A   K   L   Y   L  40

121  CCA CCA CCT ACA CCA GTC ACA CAA TTT CTT GAC ACG GAT GAC TTT GTG ACA CGC ACT GAC 180
41    P   P   P   T   P   V   T   Q   F   L   D   T   D   D   F   V   T   R   T   D  60

181  ATC TTT TAC CAC ACA AGC AGT GAC CGC TTG CTA TTT GTT GGC CAT CCA TAT TTT GAC CTG 240
61    I   F   Y   H   T   S   S   D   R   L   L   F   V   G   H   P   Y   F   D   L  80

241  AAA AAA GGA GGA AAC ACT GTA GTG CCA AAG GTT TCT GGC AGT CAA TTC AGA GTG TTT CGA 300
81    K   K   G   G   N   T   V   V   P   K   V   S   G   S   Q   F   R   V   F   R 100

301  ATG AAA TTT CCT GAC CCA AAT AAA TTC AGC TTT CAA TCT CCC TCT GTA TAT AAC CCT GAT 360
101   M   K   F   P   D   P   N   K   F   S   F   Q   S   P   S   V   Y   N   P   D 120

361  AAT CAG AGA TTA GTG TGG GCT GTA AGA GGC ATA GAA ATA TGT AGA GGA CAA CCT TTA GGA 420
121   N   Q   R   L   V   W   A   V   R   G   I   E   I   C   R   G   Q   P   L   G 140

421  GTA GGT GTT ACA GGG CAT CCA GCA TTT AAT AAG TTC AAG GAT GCT GAA AAC ATA AAT AGC 480
141   V   G   V   T   G   H   P   A   F   N   K   F   K   D   A   E   N   I   N   S 160

481  AAT TCT AAT CAA GGG GAG GAT GAT AGG GTT AAT GTA TGT GTG GAC CCC AAG CAA GTG CAG 540
161   N   S   N   Q   G   E   D   D   R   V   N   V   C   V   D   P   K   Q   V   Q 180

541  CCT TTT ATT GTG GGC TGT GTA CCA TGT GAT GGA GAG CAC TGG GAT AAA GCT ACA CCG TGC 600
181   P   F   I   V   G   C   V   P   C   D   G   E   H   W   D   K   A   T   P   C 200

601  CCC ACT GCA GAC ACA CAG CCA GGA GAT TGC CCA CCT ATT GAG CTG AAA AAC ACT AAA ATT 660
201   P   T   A   D   T   Q   P   G   D   C   P   P   I   E   L   K   N   T   K   I 220

661  CAA GAT GGA GAA ATG TGT GAT ACA GGG TGG GGC AAT TTA AAT TTT GCT ACT TTG CAG GCT 720
221   Q   D   G   E   M   C   D   T   G   W   G   N   L   N   F   A   T   L   Q   A 240

721  AGT AAG TCT GGT GTG CCC CTA GAT ATT GTT AAT CAA ACT GTG AAA TAC CCA GAT TTT TTA 780
241   S   K   S   G   V   P   L   D   I   V   N   Q   T   V   K   Y   P   D   F   L 260

781  AAA ATG GGG AGT GAT CCC TAT GGT AAT TCT ATG TTC TTT TAT GCA AAA CGA GAG CAA ATG 840
261   K   M   G   S   D   P   Y   G   N   S   M   F   F   Y   A   K   R   E   Q   M 280

841  TAT GTG AGG CAT TTG TGG GCA AGA GCA GGA ACT GTA GGG GAT GAC ATA CCT CCT GAT GGG 900
281   Y   V   R   H   L   W   A   R   A   G   T   V   G   D   D   I   P   P   D   G 300

901  GGA TAT TTT CTT TCG GGG GCA GCT AGA AGT CCT CTA CCT TCC TCA GTG TAC GTG GGT AGC 960
301   G   Y   F   L   S   G   A   A   R   S   P   L   P   S   S   V   Y   V   G   S 320

961  CCT AGT GGA TCT TTA GTT TCC AGC GAT CAG CAA ATC TAT AAT AGA CCA TTT TGG ATA CAA 1020
321   P   S   G   S   L   V   S   S   D   Q   Q   I   Y   N   R   P   F   W   I   Q 340

1021 AGG GCT CAA GGG GGA AAT AAT GGC ACT TGC TGG AAT AAT GAG CTT TTT GTA ACC GCG GTT 1080
341   R   A   Q   G   G   N   N   G   T   C   W   N   N   E   L   F   V   T   A   V 360

1081 GAT AGC ACC CGT GGT ACA AAT TTC AGT ATA TCT GTT CAC AAA GAT AAT CCC GAA GCT GGG 1140
361   D   S   T   R   G   T   N   F   S   I   S   V   H   K   D   N   P   E   A   G 380

1141 CCT CAG GAC ACC TAC AAA GCT GCA GAT TAT AAG CAT TAT TTG AGG CAT GTT GAG GAA TGG 1200
381   P   Q   D   T   Y   K   A   A   D   Y   K   H   Y   L   R   H   V   E   E   W 400

1201 GAG GTG TCC TTA GTT ATG CAG CTT TGT ATT GTG GAT CTA AAA CCA GAA TCT TTA GCT TAC 1260
401   E   V   S   L   V   M   Q   L   C   I   V   D   L   K   P   E   S   L   A   Y 420

1261 CTG CAC AAC ATG AAT TCC AGT ATT ATT GAA AAC TGG AAT TTA GGT TTT ATT CAG CCT CCA 1320
421   L   H   N   M   N   S   S   I   I   E   N   W   N   L   G   F   I   Q   P   P 440

1321 AAC AAT ATA GAA GAC CAT TAC AGA TTT ATA GAT TCA TTG GCT ACC CGT TGT CCC AAA AAA 1380
441   N   N   I   E   D   H   Y   R   F   I   D   S   L   A   T   R   C   P   K   K 460
```

1381 **TCA GAT TTG CAG GAA AAA GAG GAC CCT TAT AAA GAC ATG AAG TTT TGG GAT GTA GAT TTA** 1440
461 S D L Q E K E D P Y K D M K F W D V D L 480

1441 **ACT GAG AAA TTT TCC ATG AAT CTA GAG CAG CAT TCT CTG GGG AGA AAA TTT TTG TTT CAA** 1500
481 T E K F S M N L E Q H S L G R K F L F Q 500

1501 **ATA GGC AGA AGA GCT AGC AAA CGG TCT GCA CCG AAA TCG GTC ACA TTT GAA AGT AGT AAA** 1560
501 I G R R A S K R S A P K S V T F E S S K 520

1561 **GGA AAA AAA GCG CCA AAG CGT AGG CGG AAA AAT GTT TAG** 1599
521 G K K A P K R R R K N V * 532

ANEXO B: Código das bases degeneradas

R	K	S	W	M	Y	D	V	B	H	N
G A	G T	G C	A T	A C	T C	G A T	G A C	G T C	A T C	G A T C

ANEXO C: Normas de Publicação do periódico *Braz J Med Biol Res*

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- Poirier P, Lemieux I, Mauriege P, Dewailly E, Blanchet C, Bergeron J, et al. Impact of waist circumference on the relationship between blood pressure and insulin: the Quebec Health Survey. Hypertension 2005; 45: 363-367.
- The Cardiac Society of Australia and New Zealand. Clinical exercise stress testing. Safety and performance guidelines. Med J Australia 1996; 164: 282-284.

Abstract. Up to the first 6 authors followed by et al., Title, Journal (abbreviation), Year, Volume, Complete Pages (Abstract).

- Lima SM, Bonci DM, Grotzner SR, Ribeiro CA, Ventura DF. Loss of amacrine cells in MeHg-treated retinae in a tropical fish. Invest Ophthalmol Vis Sci 2003; 44: E-5172 (Abstract).

Article accepted for publication but not yet published. Up to the first 6 authors followed by et al., Title, Journal (abbreviation), Year of expected publication, (in press) at the end of the citation.

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- Norman IJ, Redfern SJ. Mental health care for elderly people. New York: Churchill Livingstone; 1996.

Book, chapter. Authors, Chapter Title, Editors, Book title, Edition, City, Publisher, Year, Pages of citation.

- Kintzios SE. What do we know about cancer and its therapy? In: Kintzios SE, Barberaki MG (Editors), Plants that fight cancer. New York: CRC Press; 2004. p 1-14.
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- Statistical Package for the Social Sciences (SPSS). Version 12.0. [Computer program]. Chicago: SPSS Inc.; 2006.

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